Gene Deletion of Protein Tyrosine Phosphatase 1B Protects Against Sepsis-Induced Cardiovascular Dysfunction and Mortality

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Objective—Cardiovascular dysfunction is a major cause of mortality in patients with sepsis. Recently, we showed that gene deletion or pharmacological inhibition of protein tyrosine phosphatase 1B (PTP1B) improves endothelial dysfunction and reduces the severity of experimental heart failure. However, the cardiovascular effect of PTP1B invalidation in sepsis is unknown. Thus, we explored the beneficial therapeutic effect of PTP1B gene deletion on lipopolysaccharide (LPS)-induced cardiovascular dysfunction, inflammation, and mortality.

Approach and Results—PTP1B−/− or wild-type mice received LPS (15 mg/kg) or vehicle followed by subcutaneous fluid resuscitation (saline, 30 mL/kg). α1-dependent constriction and endothelium-dependent dilatation, assessed on isolated perfused mesenteric arteries, were impaired 8 hours after LPS and significantly improved in PTP1B−/− mice. This was associated with reduced vascular expression of interleukin-1β, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, cyclooxygenase-2, and inducible nitric oxide synthase mRNA. PTP1B gene deletion also limited LPS-induced cardiac dysfunction assessed by echocardiography, left ventricular pressure–volume curves, and in isolated perfused hearts. PTP1B−/− mice also displayed reduced LPS-induced cardiac expression of tumor necrosis factor-α, interleukin-1β, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and Gp91phox, as well as of several markers of cellular infiltration. PTP1B deficiency also reduced cardiac P38 and extracellular signal–regulated protein kinase 1 and 2 phosphorylation and increased phospholamban phosphorylation. Finally, PTP1B−/− mice displayed a markedly reduced LPS-induced mortality, an effect also observed using a pharmacological PTP1B inhibitor. PTP1B deletion also improved survival in a coeliac ligation puncture model of sepsis.

Conclusions—PTP1B gene deletion protects against septic shock-induced cardiovascular dysfunction and mortality, and this may be the result of the profound reduction of cardiovascular inflammation. PTP1B is an attractive target for the treatment of sepsis. (Arterioscler Thromb Vasc Biol, 2014;34:00-00.)

Key Words: inflammation ■ nitric oxide synthase type III

Septic shock is a major healthcare problem, inducing high mortality in intensive care units.1 It is defined as a systemic inflammatory response to infection with profound effects on all organs and most notably the cardiovascular system.2 Sepsis-induced cardiovascular dysfunction is one of the major predictors of morbidity and mortality of sepsis.3 Endotoxemia, which occurs during Gram-negative bacterial infection, is involved directly in the outbreak of systemic inflammatory response. Stimulation of endothelial cells with lipopolysaccharide (LPS) or bacteria leads the upregulation of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), production of cytokines (tumor necrosis factor TNF-α, interleukin-1β [IL-1β], and IL-6),4 as well as expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) that both aggravate inflammation and impair vascular reactivity to vasoconstrictors.

Sepsis may be characterized by deregulation of the host immune system with resultant damage at the cellular and tissue levels, leading to organ failure and death. The overwhelming inflammatory response to sepsis has been attributed to alterations of multiple intracellular pathways, including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways, which further promotes cardiovascular dysfunction and damage.5,6 PI3K pathway plays a critical role in immunologic defense mechanisms acting...
in part as a compensatory mechanism in response to activation of intracellular proinflammatory signaling pathways.⁶

Among the early events that trigger inflammation, endothelial dysfunction, and especially alteration of endothelial production of nitric oxide (NO) by endothelial NO synthase (eNOS) seems to play a major role. Endothelial NO opposes many of the molecular events associated with inflammation, such as oxidative stress and expression of cytokines and adhesion molecules.⁴ In septic shock, the deleterious role of impaired NO production is illustrated by the observation that endothelial-specific overexpression of eNOS improves hemodynamics and increases survival after LPS.⁷ Thus, in this disease, the role of NO is biphasic (ie, an early impairment of endothelial production followed by a delayed overproduction of NO via iNOS expression). Based on this, endothelial protection, and especially restoration of endothelial NO production, seems as an attractive approach to counteract inflammation and ultimately reduce organ damage and mortality in sepsis.

One approach, developed in our laboratory, to restore endothelial NO production in diseases is based on inhibition of protein tyrosine phosphatase 1B (PTP1B).⁸ We demonstrated that PTP1B inhibitors, by favoring tyrosine phosphorylation, restore altered endothelial NO (eNOS) production in chronic heart failure.⁹ We also showed that long-term pharmacological inhibition or gene deletion of PTP1B reduces left ventricular (LV) dysfunction and adverse LV remodeling in chronic heart failure.¹⁰ Improvement of endothelial dysfunction after inhibition of PTP1B was related to restored PI3K/Akt/eNOS signaling, consistent with the known role of this enzyme in regulating insulin signaling¹¹ (eg, insulin receptor, insulin receptor substrate-1, PI3-K/Akt, and MAPK). In parallel, PTP1B invalidation reduces oxidant tone and improves vascular function in a mouse model of obesity,¹² and PTP1B expression is enhanced by inflammatory mediators such as TNF-α.¹⁰,¹³ Thus, PTP1B inhibition may represent an important approach for the treatments of sepsis-induced inflammation and dysfunction.

Therefore, the main objective of our study was to determine the effect of PTP1B gene deletion on cardiovascular inflammatory processes, vascular and myocardial dysfunction, and survival in LPS-challenged mice.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**PTP1B Gene Deletion in LPS Mice Reduces Smooth Muscle Contractile Dysfunction and Improves NO-Mediated Vasodilation**

The contraction induced by phenylephrine 10⁻⁷ mol/L and 10⁻⁶ mol/L was reduced in wild-type (WT) LPS mice at H-8, and this contraction was significantly improved by the selective iNOS inhibitor 1400W. This impaired contractile response was reversed significantly in PTP1B−/− mice (Figure 1A). At H-8, compared with sham, LPS mice displayed a marked impairment of flow-mediated dilatation (FMD). No-nitro-l-arginine (N-NNA) abolished FMD in sham arteries, suggesting that it is mediated entirely by NO, but did not affect FMD in arteries isolated from LPS mice, suggesting that LPS abolished flow-induced NO-mediated dilatation (Figure 1B). The LPS-induced impairment of FMD was not found in PTP1B−/− LPS H-8 mice. In those mice, N-NNA abolished FMD, suggesting that the increased FMD is mostly because of restored (endothelial) NO production (Figure 1B). In contrast, in mesenteric arteries isolated from sham mice, PTP1B deficiency reduced FMD as previously described (Figure 1B).¹¹ No significant difference in the endothelium-independent dilation to the NO donor sodium nitroprusside was found between groups (data not shown).

In another set of arteries, we found that in vitro incubation with TNF-α impaired mesenteric artery FMD and that this impairment was absent in PTP1B−/− mice. Incubation with N-NNA abolished FMD in WT and PTP1B−/− mice before and after TNF-α incubation (Figure 1C).

In parallel, in mesenteric arteries isolated from WT LPS mice, we observed a significant decrease in Akt and eNOS phosphorylation at H-8, which was significantly less marked in arteries isolated from PTP1B−/− LPS mice (Figure 1D). The changes in eNOS phosphorylation were not accompanied by significant changes in eNOS expression, although there was a tendency for a transient increase at H-4 in both WT and PTP1B−/− mice. PTP1B deficiency did not affect eNOS expression at any time point (Figure 2D). In PTP1B−/− LPS mice, the early increase of Akt phosphorylation was also found 24 hours after LPS (WT: 0.44±0.06; PTP1B−/−: 0.74±0.07).

**In Vitro and In Vivo PTP1B Inhibition Restores NO-Mediated Vasodilation in LPS and Cecal Ligation and Puncture Mice**

Acute in vitro incubation with the PTP1B inhibitor AS279 markedly improved FMD in arteries isolated from LPS mice.
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(Figure 2A) and cecal ligation and puncture (CLP) mice (Figure 2B). This improvement was inhibited significantly by l-NNA, suggesting that acute PTP1B inhibition restored NO production during endotoxemia. A similar protective effect on NO-dependent, flow-mediated vasodilation was found after a 7 days in vivo treatment with the PTP1B inhibitor AS279 (Figure 2C), showing that pharmacological blockade of PTP1B affords similar protection as that observed in PTP1B−/− mice.
PTP1B Gene Deletion Limits LPS-Induced Cardiovascular Inflammation

**Vascular Inflammation**

In WT mice, LPS enhanced the mesenteric artery expression of PTP1B, as well as of the proinflammatory genes TNF-\(\alpha\), IL-1\(\beta\), ICAM-1, VCAM-1, iNOS, COX-2, and Gp91phox and decreased expression of eNOS and COX-1. This proinflammatory profile was more marked at H-4 than at H-8 and was not associated with detectable changes in cluster of differentiation (CD) 45 expression, suggesting an absence of increased infiltration of inflammatory cells. PTP1B gene deletion limited this mesenteric artery inflammation at least at H-4, as shown by the reduced mRNA expression of TNF-\(\alpha\), IL-1\(\beta\), IL-1\(\beta\), ICAM-1, VCAM-1, iNOS, COX-2, and Gp91phox at H-4 but not at H-8, whereas no significant differences were found for eNOS and COX-1. Moreover, in WT mice, LPS induced a significant increase in blood electron paramagnetic resonance signal for hemoglobin–nitric oxide at H-8, which was less marked in PTP1B\(^{-/-}\) mice, indirectly suggesting a decreased concentration of NO (presumably linked to the decreased iNOS expression; Figure 3).

**Cardiac Inflammation**

In WT mice, LPS significantly increased the cardiac expression of PTP1B, of the proinflammatory genes TNF-\(\alpha\), IL-1\(\beta\), and Gp91phox (Figure 4A), as well as of VCAM-1, ICAM-1, and the specific markers of leukocyte (and especially macrophage) infiltration CD45, F4/80, CD11c, and CD68, and this proinflammatory profile was more marked at H-4 than at H-8 (Figure 4B). PTP1B gene deletion attenuated LPS-induced cardiac inflammation as shown by the significantly reduced expression of TNF-\(\alpha\), CAM-1, F4/80, and CD68 at H-4 and IL-1\(\beta\), Gp91phox, VCAM-1, CD45, and CD11c at H-4 and H-8 (Figure 4A and 4B).

To understand further the mechanisms by which PTP1B gene deletion negatively regulates LPS-induced inflammation, we evaluated its effect on activation (phosphorylation) of extracellular signal–regulated protein kinases 1 and 2 and p38MAPK. In WT mice, LPS significantly increased phosphorylation of p38 MAPK and extracellular signal–regulated protein kinases 1 and 2 at H-4, and these increases were significantly less marked in PTP1B\(^{-/-}\) mice (Figure 4A).
PTP1B Gene Deletion Limits LPS-Induced Impairment of Hemodynamics and Cardiac Function

Results on the evaluation of blood pressure and heart rate in conscious mice are shown in Table 1. PTP1B deletion did not affect blood pressure or heart rate in sham mice, although there was a tendency toward increased systolic pressure, in agreement with previous results. In WT mice, LPS significantly reduced systolic and pulsed pressure, without affecting diastolic or mean pressure. In LPS mice, PTP1B gene deletion prevented the reduction in systolic and pulsed pressure, and tended to prevent the decrease in diastolic pressure.

Figure 3. Vascular inflammation and nitric oxide (NO) production. Mesenteric artery mRNA expression of protein tyrosine phosphatase 1B (PTP1B), tumor necrosis factor (TNF-α), interleukin (IL)-1β, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), cluster of differentiation (CD) 45, endothelial NO synthase (eNOS), inducible NO synthase (iNOS), cyclooxygenase-1 (COX-1), COX-2, and Gp91phox (reverse transcription polymerase chain reaction), and circulating hemoglobin–nitric oxide (HbNO; electron paramagnetic resonance [EPR]; n=7–12 mice per group). †P<0.05, ††P<0.01, †††P<0.001 vs wild type (WT) sham; *P<0.05, **P<0.01, ***P<0.001 vs time-matched WT.
Figure 4. Cardiac inflammation and cellular infiltration. A, Cardiac mRNA expression of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, protein tyrosine phosphatase 1B (PTP1B), and Gp91phox (reverse transcription polymerase chain reaction; n=7–12 mice per group) and phosphorylation of extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) and P38 mitogen-activated protein kinase (MAPK; Western blot, n=4–6 mice per group). B, Cardiac mRNA expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and cluster of differentiation (CD) 45, F4/80, CD11c, and CD68 (n=7–12 mice per group). *P<0.05, ††P<0.001 vs wild type (WT) sham; **P<0.01 vs time-matched WT.
deletion significantly blunted the decrease in systolic and pulsed pressure.

Echocardiographic analysis performed in sham mice only showed a significantly lower LV end-diastolic diameter in PTP1B−/− mice versus WT, without any changes in LV fractional shortening (Table 1). In WT mice, LPS induced a significant myocardial depression as shown by the enlarged LV end-systolic diameter, decreased fractional shortening, cardiac index, rate-corrected velocity of circumferential fiber shortening, aortic maximum velocity, and impaired LV hemodynamics assessed via either load-dependent (dP/dt max, dP/dt min, LV end-systolic pressure, and τ) or load-independent parameters (LV end-diastolic pressure–volume relationship and LV end-systolic pressure–volume relationship). In LPS injected mice (H-8), compared with WT, PTP1B gene deletion was associated with a significant improvement of cardiac functional and hemodynamic parameters (Figure 5A; Table 1).

Intrinsic myocardial function was assessed ex vivo in an isolated heart system with either 1.4 or 2.5 mmol/L calcium in the solution. In sham mice, no differences of LV developed pressure were observed between WT and PTP1B−/− (Figure 5B). In WT mice, LPS significantly impaired intrinsic cardiac contractile performance as shown by the decreased LV developed pressure (Figure 5B) and its first derivative (data not shown). Compared with WT, PTP1B−/− mice showed a significant improvement of contractile performance at both calcium concentrations (Figure 5B). In addition, PTP1B deficiency significantly reduced τ (Figure 5B) both in sham and LPS mice, suggesting that it improved LV relaxation. LV relaxation largely depends on phospholamban (PLN) phosphorylation, leading to sarcoplasmic reticulum Ca2+ ATPase (SERCA) activation.15,16 Compared with WT sham, PTP1B deficiency was associated with a higher PLN phosphorylation (Figure 5C). After LPS injection, this phosphorylation was significantly reduced both in WT and PTP1B−/− mice; however, the reduction was less marked in PTP1B−/− than in WT mice. Moreover, mRNA expression of the catalytic subunits of the phosphatases PP1 and PP2A, known to play a crucial role in PLN dephosphorylation, was significantly increased by LPS in WT mice but not in PTP1B−/− mice. The expression of SERCA2a was not affected by PTP1B gene deletion or by LPS injection. Thus, the improved relaxation together with increased PLN phosphorylation strongly suggests an increased Ca2+ sarcoplasmic reticulum (SR) uptake mediated by an increased SERCA activity in PTP1B−/− mice.

**Table 1. Assessment of Blood Pressure and Heart Rate in Conscious Mice and Left Ventricular Function and Hemodynamics in Anesthetized Mice**

<table>
<thead>
<tr>
<th></th>
<th>WT Sham</th>
<th>PTP1B−/− Sham</th>
<th>WT LPS H-8</th>
<th>PTP1B−/− LPS H-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conscious mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>131±2</td>
<td>143±8</td>
<td>116±6*</td>
<td>132±3†</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>93±3</td>
<td>97±6</td>
<td>99±5</td>
<td>103±2</td>
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<tr>
<td>MBP, mm Hg</td>
<td>105±3</td>
<td>112±7</td>
<td>105±5</td>
<td>112±2</td>
</tr>
<tr>
<td>PP, mm Hg</td>
<td>38±2</td>
<td>46±3</td>
<td>16±2†</td>
<td>29±3§</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>509±25</td>
<td>570±26</td>
<td>540±13</td>
<td>535±21</td>
</tr>
<tr>
<td>Anesthetized mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.74±0.4</td>
<td>3.53±0.03*</td>
<td>3.82±0.04</td>
<td>3.62±0.07</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>1.88±0.04</td>
<td>1.81±0.04</td>
<td>3.16±0.06*</td>
<td>2.76±0.01†</td>
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<tr>
<td>FS, %</td>
<td>49.7±0.6</td>
<td>48.7±0.8</td>
<td>17.3±0.9*</td>
<td>24.0±1.2†</td>
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<tr>
<td>AoV max, cm/s</td>
<td>78.0±2.1</td>
<td>81.0±1.5</td>
<td>39.9±1.5*</td>
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<tr>
<td>Vcfc, cm/s</td>
<td>0.090±0.002</td>
<td>0.090±0.002</td>
<td>0.032±0.002*</td>
<td>0.045±0.002§</td>
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<tr>
<td>LVESP, mm Hg</td>
<td>99±5</td>
<td>100±2</td>
<td>83±2*</td>
<td>92±2†</td>
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<tr>
<td>LVEDP, mm Hg</td>
<td>3.2±0.3</td>
<td>3.3±0.4</td>
<td>1.8±0.5*</td>
<td>1.8±0.5*</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>104±7</td>
<td>106±1</td>
<td>98±2</td>
<td>105±4</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>73±5</td>
<td>75±2</td>
<td>82±2*</td>
<td>84±4*</td>
</tr>
<tr>
<td>MBP, mm Hg</td>
<td>83±6</td>
<td>85±2</td>
<td>88±2</td>
<td>91±4</td>
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<tr>
<td>PP, mm Hg</td>
<td>32±3</td>
<td>31±1</td>
<td>16±2†</td>
<td>21±2†</td>
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<tr>
<td>HR, bpm</td>
<td>452±20</td>
<td>445±21</td>
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<tr>
<td>Body weight, g</td>
<td>28.5±2.0</td>
<td>28.7±2.0</td>
<td>27.5±1.0</td>
<td>28.2±1.0</td>
</tr>
</tbody>
</table>

n=8 to 12 mice per group for conscious mice and n=5 to 6 for anesthetized mice. AoV max indicates aortic velocity maximum; DBP, diastolic blood pressure; FS, fractional shortening; HR, heart rate; LPS, lipopolysaccharide; LVEDD, left ventricular end-diastolic diameters; LVESD, left ventricular end-systolic diameters; LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; MBP, mean blood pressure; PP, pulsed pressure; PTP1B, protein tyrosine phosphatase 1B; SBP, systolic blood pressure; Vcfc, rate-corrected velocity of circumferential fiber shortening; and WT, wild type. *P<0.05 and ‡P<0.01 vs WT sham; †P<0.05 and §P<0.01 vs WT LPS H-8.
In situ mitochondrial respiration was assessed in permeabilized heart fibers. There was no difference between respiratory control ratio in L-glutamate+L-malate ADP-stimulated respiration between groups (Table 2). LPS significantly reduced respiratory control ratio in palmitoyl-L-carnitine+L-malate ADP-stimulated respiration in WT mice, and this was not affected by PTP1B gene deletion (Table 2).

PTP1B Gene Deletion Improves Locomotor Activity in Sepsis

After LPS injection, WT and PTP1B−/− mice displayed typical signs of murine endotoxic shock such as ruffled fur, diarrhea, and ocular exudates. Table 3 shows that LPS significantly reduced horizontal and vertical locomotor activities, total and vertical movement time, and the total distance covered. PTP1B deletion improved locomotor activity as shown by higher horizontal activity, total movement time, and the total distance covered during the 24-hour period after LPS injection (Table 3).

PTP1B Gene Deletion Increases Survival

Compared with WT mice, PTP1B−/− mice exhibited a significantly higher survival rate (70%) 96 hours after LPS challenge because at this time no WT mice had survived (Figure 6A). The PI3K inhibitor wortmannin increased mortality in WT mice and abolished the beneficial effect observed in PTP1B−/− mice (Figure 6B).

LPS mice pretreated for 7 days with the pharmacological PTP1B inhibitor AS279 displayed an increased survival rate compared with untreated LPS mice (Figure 6C), although this was not as marked as that observed in PTP1B−/− mice. Finally, PTP1B deficiency also significantly increased survival in CLP mice, although this effect was not as marked as that observed in LPS mice (Figure 6D). In contrast, the pharmacological PTP1B inhibitor AS279 did not affect survival of CLP mice (Figure 6E).

Discussion

The main results of our study performed in mice are as follows:
Cardiac and vascular PTP1B expression was increased in response to LPS. 

2. PTP1B gene deletion significantly reduced LPS-induced proinflammatory processes, restored endothelial function, and reduced cardiac contractile dysfunction.

3. In vivo and in vitro pharmacological inhibition of PTP1B also restored endothelial function in LPS mice.

4. Both gene deletion and pharmacological inhibition of PTP1B reduced LPS-induced mortality, and PTP1B gene deletion also improved survival in CLP mice.

Modulation of LPS-Induced Vascular Endothelial Dysfunction and Inflammation

Vascular (and especially endothelial) dysfunction is considered as a precursor of tissue damage and end-organ dysfunction. Using polymerase chain reaction experiments, our data confirmed the presence of PTP1B in the vascular wall and demonstrated that PTP1B expression was enhanced by LPS injection. Consistent with our hypothesis, PTP1B gene deletion afforded vascular protection by negatively regulating the inflammatory response to LPS. In PTP1B−/− mice, LPS-induced enhanced vascular expression of TNF-α, IL-1β, ICAM-1, VCAM-1, iNOS, COX-2, and Gp91phox mRNA was significantly reduced. ICAM-1 and VCAM-1 are known to mediate neutrophil infiltration and promote lung injury in LPS-challenged mice. Similarly, COX-2–derived metabolites participate to cardiovascular dysfunction and inhibition of this enzyme attenuates LPS-induced cardiovascular dysfunction. Thus, cytokines, enzyme-generated reactive oxygen species, and COX trigger an acute inflammatory response and induce vascular damage that may be reduced by PTP1B deletion (Figure 7).

During sepsis, vascular hyporeactivity has been mainly related to NO overproduction via iNOS expression, as confirmed by the improved contractile response to phenylephrine by the iNOS inhibitor 1400W in mesenteric arteries isolated from WT LPS mice. The higher ex vivo contractile response to phenylephrine found in PTP1B−/− mice demonstrates the beneficial effect of PTP1B gene deletion on smooth muscle contractility, probably related to the decreased vascular iNOS-mediated NO production. This is also supported by the observations of a decreased iNOS gene expression and decreased plasma hemoglobin–nitric oxide electron paramagnetic resonance signal, which indirectly suggest a decreased concentration of NO. These findings are important because iNOS expression is one of the most deleterious events leading to vascular injury and organ hypoperfusion during systemic inflammation.

Convincing evidence suggests that early endothelial dysfunction, and especially alteration of eNOS-mediated production of NO, plays a major role in triggering inflammation, ultimately contributing to end-organ dysfunction. The markedly impaired FMD observed in mesenteric arteries isolated from WT LPS mice clearly indicates severe endothelial dysfunction, whereas the abolition of the e-NNA–sensitive component of FMD reflects a profound alteration of physiological responses in PTP1B−/− mice.
NO production in these mice. In these conditions, the restoration of a large 1-NNA-sensitive FMD in PTP1B-deficient LPS mice, as well as in LPS or CLP mice, pretreated in vivo by a PTP1B inhibitor demonstrates that it is associated with restored endothelial NO production, in agreement with our previous results obtained in chronic heart failure.11

To more precisely assess the link between vascular inflammation and endothelial dysfunction, we incubated arteries with TNF-α, known to promote endothelial dysfunction ex vivo or in vivo.20 The observation that the alteration of NO-dependent FMD induced by TNF-α is blunted in PTP1B-deficient mice strongly supports the hypothesis that this intervention directly acts on cytokine-mediated endothelial dysfunction in LPS-induced sepsis.

One mechanism of this inflammation-induced endothelial dysfunction is via direct alterations of eNOS pathway, and especially of the Akt-mediated eNOS serine phosphorylation.21 We confirmed that LPS not only markedly downregulated eNOS expression but also significantly reduced the phosphorylation of Akt and of eNOS pathway in mesenteric arteries from WT mice. In PTP1B−/− mice, eNOS phosphorylation was restored, and this improvement was associated with the recovery of Akt phosphorylation. Akt-dependent phosphorylation of eNOS is necessary for a full activation of eNOS and endothelium-dependent dilatation.22 This is consistent with previous data showing that statins restored sepsis-induced impairment of Akt/eNOS phosphorylation in association with improved survival in a rabbit LPS-induced septic model.23

**Modulation of LPS-Induced Cardiac Inflammation and Contractile Dysfunction**

We found that LPS reduced arterial pressure in conscious mice and impaired LV function as shown by reduced fractional shortening, cardiac index, rate-corrected velocity of circumferential fiber shortening, and dP/dt max/min but also the load-independent indicators of systolic and diastolic dysfunction LV end-systolic pressure–volume relationship and LV end-diastolic pressure–volume relationship obtained by invasive pressure–volume relationship. These cardiovascular effects of LPS injection are consistent with previous reports.24,25 We demonstrated that PTP1B gene deletion improves arterial pressure in conscious mice, as well as both load-dependent (fractional shortening, cardiac index, rate-corrected velocity of circumferential fiber shortening and dP/dt max/min) and load-independent LV functional parameters (LV end-systolic pressure–volume relationship and LV end-diastolic pressure–volume relationship). Moreover, ex vivo perfused heart experiments revealed the beneficial effect of PTP1B gene deletion on intrinsic LV contraction (LV developed pressure and relaxation (relaxation constant τ), which thus suggests an improved intrinsic contractile status, independent of possible neurohumoral changes or modifications of load conditions.

There are different possible mechanisms for this beneficial cardiac effect during LPS-induced sepsis. Current evidence suggests that enhanced production of proinflammatory cytokines, including TNF-α and IL-1β, mediate sepsis-induced cardiac dysfunction.26 Treatment with human recombinant TNF-α and IL-1β synergistically induced reduction of myocyte contractility in vitro, and the myocardial depressant activity of human serum from septic patients was attenuated by immunoprecipitation of TNF-α and IL-1β.27 We found that PTP1B deletion limited the LPS-induced early and transient increases in myocardial TNF-α and IL-1β mRNA, which may thus be one of the mechanisms of its beneficial effect on cardiac function. In our experiments, the decreased cardiac cytokines expression was accompanied in PTP1B−/− mice by a downregulation of the adhesion molecules ICAM-1 and ICAM-2.

### Table 2. Cardiac Mitochondrial Respiration

<table>
<thead>
<tr>
<th>Respiration Rates</th>
<th>Glutamate Malate</th>
<th>Palmitoyl-L-carnitine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V_glu</td>
<td>V_gluADP</td>
</tr>
<tr>
<td>WT sham</td>
<td>18±2</td>
<td>98±6</td>
</tr>
<tr>
<td>PTP1B−/− sham</td>
<td>18±4</td>
<td>102±8</td>
</tr>
<tr>
<td>WT LPS H-8</td>
<td>17±2</td>
<td>96±16</td>
</tr>
<tr>
<td>PTP1B−/− LPS H-8</td>
<td>19±1</td>
<td>99±5</td>
</tr>
</tbody>
</table>

Cardiac mitochondrial respiratory parameters (n=6–8 mice per group). Rates of respiration are given in pmol/O2/s per milligram dry weight. V_glu: complex I–dependent state 2 respiration; V_gluADP: complex I–dependent state 3 respiration; V_palm: palmitoyl-L-carnitine stimulated state 2 respiration; V_palmADP: palmitoyl-L-carnitine stimulated state 3 respiration. LPS indicates lipopolysaccharide; PTP1B, protein tyrosine phosphatase 1B; RCR, respiratory control ratio; and WT, wild type.

*P<0.05 vs WT sham.

### Table 3. Twenty-Four-Hour Locomotor Activity

<table>
<thead>
<tr>
<th>Activity</th>
<th>WT Sham</th>
<th>PTP1B−/− Sham</th>
<th>WT LPS H-8</th>
<th>PTP1B−/− LPS H-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horizontal activity (beams crossed)</td>
<td>94370±13929</td>
<td>81596±9468</td>
<td>5198±560*</td>
<td>11317±1457†</td>
</tr>
<tr>
<td>Vertical activity (beams crossed)</td>
<td>21542±6179</td>
<td>15483±2815</td>
<td>189±42*</td>
<td>240±74</td>
</tr>
<tr>
<td>Movement time, s</td>
<td>2778±953</td>
<td>2455±536</td>
<td>83±10††</td>
<td>270±53‡</td>
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<tr>
<td>Vertical time, s</td>
<td>7904±1560</td>
<td>7255±1232</td>
<td>72±17*</td>
<td>92±27</td>
</tr>
<tr>
<td>Total distance, cm</td>
<td>24966±7289</td>
<td>23141±4790</td>
<td>708±104††</td>
<td>1951±353‡‡</td>
</tr>
</tbody>
</table>

n=6 mice per group. LPS indicates lipopolysaccharide; PTP1B, protein tyrosine phosphatase 1B; and WT, wild type.

*P<0.001 vs WT sham; †P<0.05 vs WT LPS H-8; ††P<0.01 vs WT LPS H-8.
VCAM-1, as well as a decreased infiltration of inflammatory leukocytes suggested by the reduced CD45, F4/80, CD11c, and CD68 expression. Circulating leukocytes such as monocytes and neutrophils are known to infiltrate cardiac tissue rapidly during inflammation and play an important role in LPS-induced multiple organ injury. However, it was found that blockade of ICAM-1 abolished LPS-induced cardiac dysfunction but did not reduce accumulation of neutrophils in the heart.27 Accumulating evidence reveals that ICAM-1 and VCAM-1 may induce myocardial injury independent of leukocytes infiltration. ICAM-1 activation has been shown to stimulate oxidative burst, alter calcium flux, and provoke MAPK activation in noncardiac cells.28,29 Interestingly, PTP1B has been shown to play a key role in the intracellular VCAM-1 signaling and thus participate to transendothelial migration during inflammatory response.30 In any case, the reduced ICAM-1 and VCAM-1 expression and reduced leukocytes infiltration probably participates to the beneficial effects on cardiac cytokines production and on cardiac function. In any case, although the present data and existing literature suggest a central role of reduced inflammation in the cardiovascular protection observed after PTP1B blockade, our results have not directly demonstrated that the reduction in inflammation is key driver for the observed protection and improvement in cardiac function.

We observed that deletion of PTP1B reduces LPS-induced p38MAPK and extracellular signal–regulated protein kinase 1 and 2 phosphorylation in the heart. Activation of MAPK is an important signaling pathway controlling LPS-induced cytokine production and plays a key role in TNF-α expression and myocardial dysfunction in sepsis.31 Extracellular signal–regulated protein kinase 1 and 2 activation induced TNF-α expression in several cell types including cardiomyocytes during sepsis.32 In parallel, LPS increased p38 MAPK phosphorylation in cultured neonatal cardiomyocytes, and inhibition of this enzyme reversed LPS-induced LV depression and decreased mortality.33 Thus, the effect of PTP1B deletion on MAPK phosphorylation may be a central mechanism of the observed decrease in myocardial inflammatory response.

Impairment of SR Ca2+ reuptake is another part of the postulated scenarios for LPS-induced cardiac contractile perturbations. SR calcium uptake is mediated by Ca2+-ATPase (SERCA2), whose activity is reversibly regulated by PLN. Dephosphorylated PLN inhibits SERCA, and phosphorylation of PLN relieves this inhibition.15,16 Thus, the effect of PTP1B deletion on MAPK phosphorylation may be a central mechanism of the observed decrease in myocardial inflammatory response.

Figure 7. Proposed scheme of the beneficial effects of protein tyrosine phosphatase 1B (PTP1B) inhibition/gene deletion. eNOS indicates endothelial NO synthase.
however, abundant evidence that inflammation alters myocardial calcium homeostasis. Especially, LPS was shown to provoke SR leak and cytoplasmic Ca\textsuperscript{2+} accumulation.\textsuperscript{34}

Decreased PLN phosphorylation, attributable in part to increased activity of the phosphatases PP1 and PP2A, is known to contribute to the impairment of myocardial contractility in sepsis.\textsuperscript{18,35} We found that LPS injection induced decreased mRNA expression of catalytic subunit of these 2 phosphatases in WT but not in PTP1B\textsuperscript{-/-} mice, and this could contribute to the changes in PLN phosphorylation and myocardial function.

We demonstrated that PTP1B deletion increases PLN phosphorylation in both sham and LPS mice. This observation, which parallels that of the decreased relaxation constant τ in isolated hearts, strongly suggests an increased Ca\textsuperscript{2+} SR uptake mediated by an improved SERCA activity in PTP1B\textsuperscript{-/-} mice, leading to improved relaxation. This may be as a result of increased sympathetic tone because previous study suggested that lacking PTP1B favors sympathoactivation by potentiation of leptin signaling.\textsuperscript{36} This increased PLN phosphorylation presumably results in improved intracellular calcium homeostasis that could contribute to the reduced LPS-induced intrinsic cardiac contractile dysfunction. However, because we have not specifically tried to assess whether modulation of PLN or SERCA 2a activity recapitulates cardiac functional recovery observed in LPS treated PTP1B\textsuperscript{-/-} mice, the direct role of the increased PLN phosphorylation on the observed functional changes is suggested but not directly demonstrated.

Another mechanism which may contribute to the improvement of cardiac function is mitochondrial protection.\textsuperscript{5} The mitochondrial permeability transition pore opening seems to be involved in sepsis-induced mitochondrial damage in the myocardium.\textsuperscript{6} In this context, we confirmed that LPS may be associated with myocardial mitochondrial dysfunction as shown by the impaired respiratory control ratio in palmitoyl-l-carnitine+-malate ADP-stimulated respiration. However, this impairment was not modified in PTP1B\textsuperscript{-/-} mice, implying that the beneficial effect of PTP1B deletion is not related to an improvement of this mitochondrial function.

Survival

Our survival studies showed that gene deletion or pharmacological inhibition of PTP1B protect the animals against LPS-induced death. PTP1B deficiency also improved survival in the CLP model of polymicrobial sepsis, although this latter effect was less marked than that observed after LPS, probably related to the fact that the CLP microbial model of sepsis with sustained infection induces more severe alterations than the LPS model consisting of a single and transient endotoxin challenge. To the best of our knowledge, no previous studies demonstrated this beneficial effect in sepsis.

Although the selectivity of the pharmacological inhibitor used may be questioned,\textsuperscript{10} we think that the combination of the double genetic/pharmacological approach with similar results (as already applied by us in chronic heart failure)\textsuperscript{11} provides a strong argument for the role of PTP1B, with the added value of the clinical relevance of pharmacological inhibition. It must be stressed, however, that the effect of pharmacological PTP1B inhibition was less marked than that of gene deletion, as evidenced by the results on survival (ie, more moderate effect of the pharmacological inhibitor in LPS and lack of effect in the more severe CLP model mice). This may have several causes, including lack of selectivity for PTP1B, incomplete inhibition of the enzyme, and pharmacokinetic considerations. Possible future evaluation of other pharmacological PTP1B inhibitors in subsequent studies may help increasing the clinical relevance of our work.

Interestingly, this beneficial effect on survival was absent after in vivo treatment with the PI3K inhibitor wortmannin. This is in line with a previous study reporting several beneficial effect of Akt overexpression after LPS injection. Taken together, these results suggest that the beneficial effect of PTP1B inhibition on survival may involve the PI3K/Akt (and possibly eNOS) pathway. However, because wortmannin per se increased mortality in WT mice, it is unclear whether the abolition of the beneficial effect of PTP1B deficiency indeed reflects a reversal of its effects or is simply the consequence of nonspecific deleterious effects of wortmannin. In addition, we have not performed mirror experiments testing whether selective Akt phosphorylation also induces cardiovascular protection and decreases mortality, thus determination of the exact role of PI3K and Akt phosphorylation in the observed effects requires further investigation.

The present study addresses the beneficial cardiovascular effects of PTP1B blockade associated with an improved survival; however, we have not addressed the changes occurring in other organs. Of note, a recent article showed that attenuation of PTP1B oxidation (which in theory should lead to increased PTP1B activity) protects against LPS-induced pulmonary edema.\textsuperscript{27} Thus, if possible deleterious effects on pulmonary permeability also occur in our model, they might limit the overall benefit of PTP1B blockade demonstrated here, especially in terms of mortality.

Conclusions

In conclusion, our study revealed for the first time that pharmacological inhibition or gene deletion of PTP1B reduced LPS-induced mortality, probably related to the reduction of cardiovascular inflammation and dysfunction. Sepsis and septic shock lead to considerable morbidity and mortality in developed and developing countries. Despite advances in understanding the innate immune events that lead to septic shock, molecular therapies based on these advances have failed to improve sepsis mortality. Taken together, our results suggest for the first time that inhibition of PTP1B may be a new pharmacological target for the treatment of sepsis.

Acknowledgments

We thank Dr Michel L. Tremblay (Goodman Cancer Centre, McGill University, Montreal, Canada) for providing us the protein tyrosine phosphatase-deficient mice, Brigitte Dautréaux for her excellent technical assistance, and Anne-Marie Lompré and Catherine Pavoine for useful scientific discussions.

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Disclosures

None.
Protein tyrosine phosphatase 1B inhibition confers cardiovascular protection during experimental chronic heart failure; however, its potential role in systemic inflammatory disease has not been assessed. Our study revealed for the first time that pharmacological inhibition or gene deletion of protein tyrosine phosphate 1B reduced lipopolysaccharide-induced mortality and cardiovascular dysfunction, probably related to the reduction of cardiovascular inflammation. These results suggest that protein tyrosine phosphatase 1B is an attractive target for the treatment of sepsis.

Significance
Gene Deletion of Protein Tyrosine Phosphatase 1B Protects Against Sepsis-Induced Cardiovascular Dysfunction and Mortality

David Coquerel, Remi Neviere, Eugenie Delile, Paul Mulder, Xavier Marechal, David Montaigne, Sylvanie Renet, Isabelle Remy-Jouet, Elodie Gomez, Jean-Paul Henry, Jean-Claude do Rego, Vincent Richard and Fabienne Tamion

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To assess the role of PI3K/Akt pathway, mice were pretreated 1 hour before LPS injection with Wortmannin, an inhibitor of PI3K (1mg/kg; i.v.). For pharmacological treatment in the LPS model, mice were either untreated or treated with the PTP1B inhibitor AS279 (Merck-Serono Pharmaceutical Research institute) at the dose of 60mg/kg/day in drinking water\(^4\), starting 7 days before LPS injection. For pharmacological treatment in the CLP model, mice were either untreated or treated with AS279 at the dose of 30mg/kg, given 10 hours after CLP induction.

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Locomotor activity was assessed automatically using a computerized actimeter (Versamax, AccuScan Instruments, Inc., Ohio, USA) which monitored horizontal displacement and vertical movements as previously described\(^5\). The animals were placed individually in 20 x 20 x 30cm compartments, immediately after LPS or 0.9% NaCl. The responses were expressed as the number of crossed beams and time in movement (s) by each mouse during a 24 hour period.

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Mesenteric arteries, 2 mm in length and <300\(\mu\)m in diameter, were cannulated at both ends and placed in a video-monitored perfusion system (Living Systems Instrumentation, Burlington, Vt) as previously described\(^6\). First, contractility was assessed by concentration-response curves to phenylephrine (Phe; \(10^{-9}\) to \(10^{-5}\) mol/L, Sigma) before and after incubation with the selective iNOS inhibitor 1400W (\(10^{-5}\)mol/L; Sigma). Vascular dilatation was assessed under 1400W incubation in preconstricted vessels (Phe \(10^{-5}\)mol/L). Endothelium-
dependent response was assessed by flow mediated-dilatation (FMD) induced by stepwise increases in intraluminal flow (0-200 µl/min). To investigate the mechanisms involved in the endothelial effect of LPS and PTP1B deletion, particularly with regard to the role of eNOS, all of the above vascular experiments were repeated in the presence of a non selective inhibitor of NOS (N-nitro-L-arginine (L-NNA), 10^{-4}mol/L, Sigma-Aldrich). Acute PTP1B inhibition was assessed by incubating the arteries with AS279 (10^{-5}mol/L). The inhibitor was added in the bath 40 min before the increase of intraluminal flow. The first curve was thus taken as control, and the effect of the inhibitor was tested on the second and third curve. In another set of arteries, FMD was assessed before and after a 40 min infusion with TNF-α (1 ng/ml) as previously described.

**Echocardiography**

Mouse echocardiography was performed in Sham and LPS mice at H-8 in isoflurane-anesthetized closed-chest mice (1%; 1.5mL/min; Baxter), using a Vivid 7 ultrasound echograph equipped with a M12L linear probe operating at 14 MHz and fitted out with Echopac PC software (GE medical). In brief, a two-dimensional short axis view of the LV was obtained at the level of the papillary muscle, and the M-mode tracing was recorded. From these recordings, LV Diastolic and Systolic Diameters (LVDD; LVSD) as well as LV diastolic and systolic wall thickness were measured by the leading edge method according to the American Society of Echocardiography guidelines. Fractional Shortening (FS) was calculated as previously described. In addition, a pulsed Doppler of the LV outflow was performed to obtain heart rate and Time Velocity Integral (TVI) in order to calculate Aortic Velocity maximum (AoVmax), Cardiac Index (CI) and rate corrected Velocity of circumferential fiber shortening (Vcfc) as previously described.

**Cardiac hemodynamics**

LV hemodynamics was assessed by pressure-volume curves in Sham and LPS mice at H-8. In brief, under isoflurane anesthesia (1%; 1.5mL/min; Baxter) and local injection of xylocain, 2mg/kg, a 1.4F miniaturized combined conductance catheter-micromanometer (model SPR-671, Millar Instruments) connected to a pressure-conductance unit (MPCU-200, Millar) was advanced retrogradely via the carotid artery into the LV, in order to measure LV End-Systolic (LVESP) and End-Diastolic (LVEDP) Pressure, LV dP/dt_{max/min}, and LV relaxation constant Tau (Weiss method). Pressure-Volume loops were recorded at baseline and during loading by gently occluding the abdominal aorta with a cotton swab allowing the calculation of LV end-systolic and end-diastolic pressure-volume relation (LVESPVR and LVEDPVR) as indicators of load-independent LV passive compliance and contractile function, respectively. Blood pressure was assessed in conscious mice at H-8 after insertion of a micromanometer (1.2F, Millar Instruments) in the right carotid artery under isoflurane anesthesia (1.5%; 1.5mL/min; Baxter) and sedation (nalbuphin, 0.75mg/kg). Mice were allowed to recover, and pressure was assessed 30 minutes after recovery from anesthesia.

**Isolated perfused heart**

Following cervical dislocation, mouse hearts were excised and immediately mounted, via the ascending aorta, onto a Langendorff apparatus and perfused at constant coronary flow (2.5 mL/min) with aerated Krebs-Henseleit bicarbonate buffer. Mechanical activity was assessed in paced hearts (9Hz) through isovolumic contraction by inserting a latex balloon into the left ventricle. The balloon was linked to a pressure transducer connected to ML118 bridge amplifier that fed into a Powerlab 8 SP high-performance data acquisition system (ADInstruments Ltd. by Phyemep, Paris, France). Left ventricular end-diastolic pressure (LVEDP) was set to 6-8 mmHg by increasing the volume of the water-filled balloon. Isovolumic contraction was appreciated by measuring left ventricular developed pressure (LVDP) and its first derivatives.

**Mitochondrial respiration**
Respiratory parameters were studied in situ in saponin-permeabilized fibers isolated from mouse LV. Freshly excised LV was placed into ice-cold biopsy preservation solution BIOPS (2.77 mmol/L CaK2EGTA, 7.23 mmol/L K2EGTA, 6.56 mmol/L MgCl2, 0.5 mmol/L dithiothreitol, 50 mmol/L K-MES, 20 mmol/L imidazole, 20 mmol/L taurine, 5.3 mmol/L Na2ATP, 15 mmol/L phosphocreatine, pH 7.1). Fiber bundles were separated and placed into BIOPS solution containing 50µg/mL saponin. After 30 min, permeabilized fibers were rinsed three times with mitochondrial respiration media MIRO5 (110 mmol/L sucrose, 20 mmol/L HEPES, 10 mmol/L KH2PO4, 20 mmol/L taurine, 3 mmol/L MgCl2 6H2O, 60 mmol/L MES-K, 0.5 mmol/L EGTA and 0.1 % bovine serum albumin; pH 7.1). Approximately 2 mg of wet fibers were placed in the O2K oxygraph (Oroboros Instruments, Innsbruck, Austria). In the first series of experiments, 10 mmol.L\(^{-1}\) L-glutamate + 2 mmol.L\(^{-1}\) L-malate were added into the chambers. The subsequent measured respiration, i.e. in absence of exogenous ADP, was referred to as state 2. Then, ADP (2.5 mmol/L) was brought to obtain the ADP-coupled respiration called state 3. In the second series of experiments, respiration was measured in the presence of palmitoyl-L-carnitine (80 µmol/L) and L-malate (2 mmol/L) followed by addition of ADP (2.5 mmol/L). As in permeabilized fibers state 4 respiration cannot be achieved because of the presence of many intracellular ATPases that avoid total ADP depletion, the ratio state 3 to state 2 (respiratory control ratio, RCR) was used to evaluate the quality of the mitochondrial coupling between oxygen consumption and phosphorylation. Respiration studies were performed at 25°C and rates were expressed in pico moles O\(_2\) per second per milligram wet weight (Datlab4 software, Oroboros).

Western Blotting
LV tissues and mesenteric arteries were homogenized by mechanical disruption in cold Phosphosafe Extraction Reagent (Novagen) lysis buffer. The amount of proteins loaded on the gel was verified by a Bradford\(^8\) assay and was in each case 25µg. The homogenized tissue was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Mini Gel Protein III System, Bio-Rad Laboratories, Hercules, USA) and transferred on Hybond ECL membranes (Amersham Biosciences) for 120 minutes at 100 V (Minitrans-blot Cell, Bio-Rad Laboratories). Membranes were incubated with the following primary antibodies: anti-phospho-eNOS (monoclonal, Alexis Biochemicals), anti-eNOS (monoclonal, BD transduction Laboratories), anti-Akt (polyclonal, Cell Signaling Technology, Danvers USA), anti-phospho-Akt (Ser473) (polyclonal, Cell Signaling Technology), anti-MAPK (polyclonal, Cell Signaling Technology), anti-phospho-MAPK (polyclonal, Cell Signaling Technology), anti-P38 (polyclonal, Santa Cruz Biotechnology, USA), anti-phospho-P38 (polyclonal, Santa Cruz Biotechnology), anti-phospholamban (polyclonal, Cell Signaling Technology), anti-phospho-phospholamban (Ser16/Thr17) (polyclonal, Cell Signaling Technology), anti-SERCA2a ATPase (monoclonal, Thermo Scientific), anti-actin (polyclonal, Santa Cruz Biotechnology). Membranes were washed again and incubated with horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, USA). Proteins were visualized with the use of a Chemiluminescence kit (Lumi Light, Roche). Densities of the specific bands were estimated on a densitometer analyser using BioCapt and Bio-Profil (Bio-ID) software. Results are presented as the fold increase over control (time point = 1) of the density band ratio of phosphorylated protein versus total protein in the same sample, respectively.

Polymerase Chain Reaction Experiments
Total mRNA was extracted from LV tissue with the TriZol reagent (Gibco life science) according to the manufacturer’s instructions and total mRNA was extracted from mesenteric arteries with Rneasy Micro kit (Qiagen). The cardiovascular expression of TNF-α, IL1-β, iNOS, COX-1, COX-2, Gp91phox, VCAM-1, ICAM-1, CD45, F4/80, CD11c, CD68, PTP1B and 18S mRNA was assessed by quantitative real-time reverse transcription-polymerase chain
reaction (RT-PCR) with a light cycler (Roche, Basel, Switzerland) using SYBR green I. The primers were obtained from Sigma-Aldrich and had the following sequences:

**TNF-α** Forward 5’-TAGCCAGGAGGAGAACAGA-3’; Reverse 5’-TTTTCCTGGAGGAGATGTGG-3’; **IL1-β** Forward 5’-CAGGCAGGCAGTATCCTCACTCA-3’; Reverse 5’-TGTTCCTCATCCTGGAAGGT-3’; **iNOS** Forward 5’-TGGTGTTGCAAGCACAATTT-3’; Reverse 5’-AAGGCCAACACACAGCATACC-3’; **COX-1** Forward 5’-TATCCTCAACTGGAAGG-3’; Reverse 5’-TGGCTGGCCTAGAATC-3’; **COX-2** Forward 5’-AGGTCTATGGTGAGGAGGGTG-3’; Reverse 5’-GGTTCTCAGGGATGTGGAGGA-3’; **eNOS** Forward 5’-GACCCTCACCAGCTAACAAT-3’; **iNOS** Forward 5’-TGGTGTTGCAAGCACAATTT-3’; Reverse 5’-AAGGCCAACACACAGCATACC-3’; **Gp91phox** Forward 5’-AAAGGTGGTCATCACCAAG-3’; Reverse 5’-ACTGTCCCACTCCATCTTG-3’; **VCAM-1** Forward 5’-CTTTACCTGTGCCGCTTGAC-3’; Reverse 5’-ACCTCCACCTGGTTCTCTCT-3’; **ICAM-1** Forward 5’-ACTGGCAGTGTGTTCTGTGCT-3’; Reverse 5’-AAAGGTGGTCATGAGG-3’; **CD45** Forward 5’-TCGTACCCCATGGGCTAT-3’; Reverse 5’-CTGTACCCCATGGGCTAT-3’; **F4/80** Forward 5’-CTGTACCCCATGGGCTAT-3’; Reverse 5’-CTGTACCCCATGGGCTAT-3’; **Cd11c** Forward 5’-AATGAAGCAGGCAGCATCCCA-3’; Reverse 5’-CTGTACCCCATGGGCTAT-3’; **Cd68** Forward 5’-GACCCTCACCAGCTAACAAT-3’; Reverse 5’-CTGTACCCCATGGGCTAT-3’; **PTP1B** Forward 5’-CTGTACCCCATGGGCTAT-3’; Reverse 5’-CTGTACCCCATGGGCTAT-3’; **PP1c** Forward 5’-AAAGGTGGTCATCACCAAG-3’; Reverse 5’-ACTGTCCCACTCCATCTTG-3’; **PP2Acα** Forward 5’-TGGTTCCTAGAGCGTGG-3’; Reverse 5’-AAGATGCTGACGAGCTGCAAG-3’; **18S** Forward 5’-CTGTACCCCATGGGCTAT-3’; Reverse 5’-CTGTACCCCATGGGCTAT-3’.

**Electronic paramagnetic resonance (EPR) studies**

In one set of animals experiments blood samples were harvested and flash frozen in liquid nitrogen for subsequent EPR assay of HbNO, which reflects the blood NO concentration. The EPR spectrum was recorded at 77K into liquid nitrogen-filled quartz dewar using an EPR X-band (94Hz) spectrometer (Miniscope MS200, Magnettech, Berlin), applying the following parameters: Field 331.8± 28.8 mT, modulation amplitude 1T, microwave power 4.0mW, gain 10^2, resolution 4096 points. The peak- to-peak amplitudes of the 2nd hyperfine components of the triplet signals were used to quantify the HbNO (As indicated in figure: ∆ (AU)).
Survival
For LPS injection, mice were randomly assigned to five groups: Sham WT, Sham PTP1B\(^{-/-}\), WT LPS, PTP1B\(^{-/-}\) LPS and WT LPS treated with the PTP1B inhibitor AS279 during seven days before survival assessment (n=20 in each group).
For CLP, mice were randomly assigned to five groups: Sham WT, Sham PTP1B\(^{-/-}\), WT CLP, PTP1B\(^{-/-}\) CLP and CLP treated with the PTP1B inhibitor AS279 (n=20 in each group).
The animals were allowed free access to water and food, and the survival time was recorded during 124 hours.

Statistical analysis
All results are given as mean ± SEM. Normality was verified using the Kolmogorov-Smirnov test. Difference between two groups was analyzed by a standard Student t-test. For multi-group comparisons, one way ANOVA followed by Newman-Keuls test or two way ANOVA followed by Bonferroni test were performed. P<0.05 was considered statistically significant.
REFERENCES


MATERIAL and METHODS

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reaction (RT-PCR) with a light cycler (Roche, Basel, Switzerland) using SYBR green I. The
primers were obtained from Sigma-Aldrich and had the following sequences:

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TTTCCTGGGAGGATGTGG-3'; **IL1-β**
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TTGTGGCCTAGAAGACTC-3'; **COX-2**
Forward 5'-AGGTCACTTTGGGAGAAGTG-3' Reverse 5'-GGTTTCTCAAGGGATGTGAGGA-3'; **eNOS**
Forward 5'-GACCCTACCCGCTACACAT-3' reverse 5'-CACAGGGATGAGGTGTTGTCCT-3'; **iNOS**
Forward 5'-TGGTGGTGCAAGCAGCATTT-3' Reverse 5'-
AAGGCCAACACACAGCATACC-3' **Gp91phox**
Forward 5'-
AAAGGTGGTCTCATACCAAGG-3'; Reverse 5'-ACTGTCCCCACCTCCATCTTG-3';
**VCAM-1**
Forward 5'-TCTTACCTGTGCGCTGTGAC-3'; Reverse 5'-
ACCTCCACCTGGTCTCTCTTT-3'; **ICAM-1**
Forward 5'-ACTGGCAGTGTTCTCTGTGCT-3'; Reverse 5'-AAAGTACGGGTGGAGAAGGT-3';
**CD45**
Forward 5'-TGGTGCCCAACAAATTACA-3'; Reverse 5'-ATCCCCAAATCTGTGTCAC-3';
**F4/80**
Forward 5'-CTGTAACCGGATGGCAAACT-3'; Reverse 5'-
ATGGTGGTGCTGTTCTGCT-3'; **Cd11c**
Forward 5'-AATGAAGCAGCCATTCATCC-3'; Reverse 5'-
GCAGCAGTGACATGGCTG-3'; **Cd68**
Forward 5'-GCAGCAGTGACATGGCTG-3'; Reverse 5'-
AATGAAGCAGCCATTCATCC-3'; **PTP1B**
Forward 5'-TCTTACCTGTGCGCTGTGAC-3'; Reverse 5'-
ATGGTGGTGCTGTTCTGCT-3'; **PP1c**
Forward 5'-CACTGGCCACCTCCATCC-3'; Reverse 5'-
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**Electronic paramagnetic resonance (EPR) studies**
In one set of animals experiments blood samples were harvested and flash frozen in liquid
nitrogen for subsequent EPR assay of HbNO, which reflects the blood NO concentration. The
EPR spectrum was recorded at 77K into liquid nitrogen-filled quartz dewar using an EPR
X-band (94Hz) spectrometer (Miniscope MS200, Magnettech, Berlin), applying the following
parameters: Field 331.8± 28.8 mT, modulation amplitude 1T, microwave power 4.0mW, gain
10², resolution 4096 points. The peak- to-peak amplitudes of the 2nd hyperfine components of
the triplet signals were used to quantify the HbNO (As indicated in figure: Δ (AU)).
Survival
For LPS injection, mice were randomly assigned to five groups: Sham WT, Sham PTP1B−/−, WT LPS, PTP1B−/− LPS and WT LPS treated with the PTP1B inhibitor AS279 during seven days before survival assessment (n=20 in each group).

For CLP, mice were randomly assigned to five groups: Sham WT, Sham PTP1B−/−, WT CLP, PTP1B−/− CLP and CLP treated with the PTP1B inhibitor AS279 (n=20 in each group).
The animals were allowed free access to water and food, and the survival time was recorded during 124 hours.

Statistical analysis
All results are given as mean ± SEM. Normality was verified using the Kolmogorov-Smirnov test. Difference between two groups was analyzed by a standard Student t-test. For multi-group comparisons, one way ANOVA followed by Newman-Keuls test or two way ANOVA followed by Bonferroni test were performed. P<0.05 was considered statistically significant.
REFERENCES


MATERIAL and METHODS

Animal experiments
Experiments were performed in 10 to 12 week-old BalbC/J (WT) for endotoxemic model and 10 week-old C57B/6 mice for CLP, purchased from Janvier Laboratories (Le Genest Saint Isle, France). For gene deletion, we used PTP1B−/− mice generated as previously described1 and bred in our laboratory. All animals were housed in plastic cages maintained on a 12-h light/dark cycle in a controlled temperature (24±2 °C) and humidity (50±5%), and allowed free access to standard mice chow and water. Investigations conformed to the directive 2010/63/EU of the European parliament. All animal protocol was approved by Haute Normandie Ethics review board (authorization 76-95).

Experimental protocol
Endotoxin shock was induced by intraperitoneal (i.p.) injection of Escherichia coli 055:B5 Lipopolysaccharide (LPS; Sigma). LPS was administered at a lethal dose of 15 mg/kg. Normal (sham-operated) mice received an equivalent volume of vehicle (0.9% saline solution). One, 5 and 9 hours later, LPS mice received subcutaneous fluid resuscitation at a dose of 30 mL/kg with 0.9% saline solution to correct the fall in blood pressure3. To assess the potential therapeutic effect of pharmacological PTP1B inhibition during polymicrobial sepsis, we used a Cecal Ligature and Puncture (CLP) model of sepsis. Following laparotomy, the cecum was exposed, ligated below the ileocecal valve and punctured twice with a 21G needle. A small amount of fecal material was extruded from the puncture site, and the cecum was then placed back into the peritoneum.

Study design
Mice were sacrificed 4 hours (H-4) and 8 hours (H-8) after LPS injection in order to collect blood, mesenteric arteries and heart. In another set of experiments, cardiovascular function was evaluated in Sham and LPS mice at H-8. Thus, we performed experiments in six groups (n= 8-12/group): WT Sham, PTP1B−/− Sham, WT LPS H-4, WT LPS H-8, PTP1B−/− LPS H-4 and PTP1B−/− LPS H-8.

Drug administration
To assess the role of PI3K/Akt pathway, mice were pretreated 1 hour before LPS injection with Wortmannin, an inhibitor of PI3K (1mg/kg; i.v.). For pharmacological treatment in the LPS model, mice were either untreated or treated with the PTP1B inhibitor AS279 (Merck-Serono Pharmaceutical Research institute) at the dose of 60mg/kg/day in drinking water4, starting 7 days before LPS injection. For pharmacological treatment in the CLP model, mice were either untreated or treated with AS279 at the dose of 30mg/kg, given 10 hours after CLP induction.

Locomotor activity
Locomotor activity was assessed automatically using a computerized actimeter (Versamax, AccuScan Instruments, Inc., Ohio, USA) which monitored horizontal displacement and vertical movements as previously described5. The animals were placed individually in 20 x 20 x 30cm compartments, immediately after LPS or 0.9% NaCl. The responses were expressed as the number of crossed beams and time in movement (s) by each mouse during a 24 hour period.

Vascular function
Mesenteric arteries, 2 mm in length and <300µm in diameter, were cannulated at both ends and placed in a video-monitored perfusion system (Living Systems Instrumentation, Burlington, Vt) as previously described5. First, contractility was assessed by concentration-response curves to phenylephrine (Phe; 10−9 to 10−5 mol/L, Sigma) before and after incubation with the selective iNOS inhibitor 1400W (10−5mol/L; Sigma). Vascular dilatation was assessed under 1400W incubation in preconstricted vessels (Phe 10−5mol/L). Endothelium-
dependent response was assessed by flow mediated-dilatation (FMD) induced by stepwise increases in intraluminal flow (0-200 µl/min). To investigate the mechanisms involved in the endothelial effect of LPS and PTP1B deletion, particularly with regard to the role of eNOS, all of the above vascular experiments were repeated in the presence of a non selective inhibitor of NOS (N-nitro-L-arginine (L-NNA), 10^{-4}mol/L, Sigma-Aldrich). Acute PTP1B inhibition was assessed by incubating the arteries with AS279 (10^{-5}mol/L).The inhibitor was added in the bath 40 min before the increase of intraluminal flow. The first curve was then taken as control, and the effect of the inhibitor was tested on the second and third curve. In another set of arteries, FMD was assessed before and after a 40 min infusion with TNF-α (1 ng/ml) as previously described.

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